Improved walnut mass micropropagation through the combined use of phloroglucinol and FeEDDHA

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Abstract Despite the socioeconomic importance of walnut trees, poor rooting and recalcitrance to in vitro culture have hampered the establishment of high-yield clonal plantations. To improve walnut micropropagation, we introduced several modifications to current methods and evaluated the effects on microshoot performance and acclimatization. Nine selected genotypes (13-year-old trees) of the commercial hybrid *Juglans major* 209 × *J. regia* were cultured in vitro on DKW-C medium supplemented with 4.4 μM BA and 50 μM IBA. A protocol was developed that relies on the sequential use of 0.4 and 0.2 mM phloroglucinol during shoot multiplication, but not at later stages, so as to maximize shoot growth without inhibiting root formation. Moreover, substituting FeEDTA by FeEDDHA diminished chlorotic symptoms and significantly improved the rooting ability of all genotypes, with up to 90 % microshoots developing viable roots at 6.81 mg/L Fe^{3+}. The addition of 83.2 mM glucose during the root expression phase was particularly efficient at promoting plant survival during acclimatization, compared to equimolar amounts of the alternative sugars sucrose and fructose. At the proposed working concentrations, the aforementioned compounds did not cause any deleterious effects on the nine genotypes studied. Microscopic analysis revealed the physical continuity between adventitious roots and stem cambial tissue. Analysis of leaf genomic DNA with eight polymorphic microsatellite markers was supportive of the clonal fidelity and genetic stability of the micropropagated material. Successful clonal plantations (over 5800 ramets) have been established by applying this protocol.

Keywords Acclimatization · Adventitious rooting · Clonal plantation · Iron source · *Juglans* · Carbon source

Introduction

The genus *Juglans* includes some of the most valuable timber species in the world, such as the common walnut (*J. regia*) or the American walnuts *J. major* (Arizona), *J. nigra* (Black) or *J. hindsii* (Northern California). Over the last decades, breeders have developed a number of hybrid varieties for improved timber production in dedicated plantations (Woeste and Michler 2011). The hybrid *J. major* 209 × *J. regia* (MJ209xRA) is one of the most frequently planted. Produced in France from open-pollinated seed orchards, it has shown both phenomenal growth and environmental plasticity under a variety of field conditions (Becquey 1997; Aletá et al. 2004; Clark and Hemery 2010; Woeste and Michler 2011). The socioeconomic potential of walnut hybrids will not be fully developed, however, until cost-efficient mass propagation methods are available for superior genotypes selected under field conditions.

Micropropagation can be a promising alternative to traditional vegetative propagation. Since the first in vitro procedures were developed for walnut (Driver and Kuniyuki 1984; McGranahan et al. 1987; McGranahan and Leslie 1988; Cornu and Jay-Allemand 1989), a number of modifications have been tested to increase success and extend the methodology to different species and hybrids (e.g., Dolcet-Sanjuan et al. 2004; Leal et al. 2007; Bosela and Michler 2008; Leslie et al. 2009; Vahdati et al. 2009; Toosi and Dilmagani 2010). To date, a standardized in vitro protocol has not been established. In addition to the problem of introducing field-selected material to in vitro culture, better procedures are needed regarding microshoot multiplication, microshoot rooting and plantlet acclimatization. Poor rooting is arguably the main factor limiting the establishment of clonal plantations (Woeste and Michler 2011). The aim of this study was to improve current micropropagation protocols using selected MJ209xRA genotypes (plus trees) from commercial seedling plantations. Preliminary data advanced by Moreno et al. (2012) led us to focus on the potential use of phloroglucinol (PG; 1,3,5-trihydroxybenzene) and Fe-ethylenediamine-N,N′-bis(2-hydroxyphenylacetate) (FeEDDHA). FeEDTA is the typical iron source in tissue culture media, whereas the most common plant growth regulators used are 6-benzylaminopurine (BA), indole-3-butyric acid (IBA), and 1-phenyl-3-(1,2,3–thiadiazol-5-yl)urea (thidiazuron). PG is a natural precursor of lignin biosynthesis with complex effects on plant tissue culture (Teixeira da Silva et al. 2013). Several reports suggest PG can mimic auxin or cytokinin action (Kumar et al. 2005; Tallón et al. 2012); and PG is effective in attenuating stress damage and removing hyperhydricity caused by poor lignification (Phan and Hegedus 1985). We report here an optimized protocol that substantially increases walnut microshoot rooting and acclimatization, compared with previous reports, without affecting the genetic stability of the micropropagated material. Nine selected genotypes have been successfully planted under a commercial setting following this methodology.

Materials and methods

Plant material

All material was obtained from commercial plantations of hybrid walnut, *Juglans major* (Torrey) Heller x *J. regia* L., located in La Vera (Extremadura, Spain). This region is characterized by a mild continental climate well suited for walnut growth, with 840 mm mean annual rainfall and 14 °C mean annual temperature (last 15-years average; National Agency of Meteorology, Spain, www.aemet.es/en). Seedlings were grown from random seeds obtained in open pollinated crosses, where *J. major* var. 209 was the maternal component. A selection program started shortly afterwards, based on the systematic scoring of phenology, morphology, and wood quality. In vitro introduction was performed with the best scoring individual trees, renamed here as J1 to J9 for convenience. Trees were 13-years-old when samples were collected in the field.

Culture conditions

Unless otherwise stated, all reagents were purchased from Duchefa Biochemie (The Netherlands). In vitro introduction was particularly efficient for dormant buds located at the basal portion of spontaneously-sprouted epicormic branches (no coppicing was performed). Branches were collected in late winter, transported to the lab in tagged plastic bags, and profusely rinsed with sterile water (3 × 10 min in an orbital shaker). After removal of apical portions (~7 cm), branch segments of 20–25 cm were disinfested for 15 min in 0.5 % (v/v) Previcur® (Bayer CropScience) to prevent fungal growth. To promote bud breaking, twigs were kept partly submerged in water (40-ml glass beakers) in growth chambers under a 16 h-light (cool-white fluorescent light; Master TL-D 58 W/840 lamps, Philips)/8 h-dark photoperiod. Water was replaced weekly. During the photoperiod, photosynthetic photon flux density (PPFD) was linearly increased from zero to 100 μmol m^{-2} s^{-1} for the first 8 h, and then decreased in identical manner for the remaining 8 h. Temperatures followed the same pattern: from 17 °C (basal) to 26 °C for 8 h and back to 17 °C in 8 h. Bud sprouting normally occurred after 3 weeks under these conditions. When shoots reached ~ 2 cm, they were excised and disinfested by soaking for 20 min in commercial bleach solution (1 % sodium hypochlorite, w/v) supplemented with 0.01 % (v/v) Tween-20, followed by extensive rinsing with sterile water (3 × 10 min in an orbital shaker). The DKW-C medium of McGranahan et al. (1987) supplemented with 4.4 μM BA, 50 μM IBA, 0.6 mM PG, and 5.5 g/L agar (Industrial agar 707469, Pronadisa, Spain) was used for the shoot establishment phase. This was conducted at 24 °C in 150 × 25 mm glass test tubes under 16 h-light (50 μmol m^{-2} s^{-1})/8 h-dark. The presence of endogenous bacteria was monitored by culturing small basal-callus samples in Petri dishes containing 5 g/L meat and yeast extract (Panreac), 5 g/L glucose, 2 g/L KH_2PO_4, and 7 g/L agar. Explants showing no contamination were subcultured every week until active growth became visible. For the next stages, 580-mL straight-sided cylindrical glass jars (124 mm high × 80 mm diameter) were used, with eight shoots being cultured per jar. PG was included or not in the DKW-C medium during the establishment and multiplication phases to evaluate its effects on microshoot performance, that is,

elongation, rooting, and acclimatization. Two alternative iron sources, FeEDTA or FeEDDHA, were also tested. Once optimal concentrations were established for PG and the iron source, three alternative carbon sources were tested during the root expression phase: sucrose, glucose or fructose, at a final concentration of 83.2 mM.

Efficient rooting was achieved in two successive steps following McGranahan et al. (1987). To promote root induction, healthy microshoots at least 2 cm in length were cultured in DKW-C medium containing one-half macronutrients, 117 mM sucrose, and 50 μM IBA (Vahdati et al. 2004; Dolcet-Sanjuan et al. 2004). IBA gave results superior to indole-3-acetic acid (IAA) or 1-naphthaleneacetic acid (NAA). After 5 days in the dark, root elongation was favored by removing the auxin and replacing agar with vermiculite (4:50, w/v; vermiculite type 3, grain size 1-4 mm, Projar, Spain), following Jay-Allemand et al. (1992), except that the gelled medium was replaced by liquid medium. This second phase took 2–3 weeks, depending on the specific genotype, and was conducted under multiplication conditions. Rooted microshoots were then potted in a mixture of peat (50/50 tray, Gramoflor, Germany) and vermiculite (3:1, v/v) and acclimatized for 8–10 additional weeks in a greenhouse (March-August). For the first 3 weeks, maximum lightning was set at about 150 μmol m^{-2} s^{-1} and relative humidity at 70–80 %. During the remaining 5-7 weeks, plants were subjected to higher PPFD (up to 450 μmol m^{-2} s^{-1}) and lower humidity (50–60 %). Pots were initially covered with transparent plastic, which was progressively punctured and loosened until complete removal at the end of week four. Greenhouse temperatures ranged from 16 to 32 °C during the entire acclimatization process.

Phloroglucinol application

PG was applied only during the establishment and multiplication phases, because of its inhibitory effect on rooting. It was tested at 0, 0.2, 0.4, 0.6, and 0.8 mM. Only apical tips with 4–6 buds (~1 cm in length) were used as explants for these experiments. Before root induction, callus weight, internodal length, and number of nodes were measured. The number of roots per microshoot, length of the longest root, and rooting percentage were scored between two (genotypes J1 and J5) and 3 weeks after shifting to root expression medium. At least 64 microshoots (8 per jar) were analyzed per genotype and replica. All experiments were independently replicated three times.

Iron source

Two different concentrations of FeEDTA or FeEDDHA (6.81 and 10.21 mg/L Fe), were added to the DKW-C medium to compare the effects on growth, rooting, and survival. At least 64 microshoots (8 per jar) were scored per genotype and replica. Three independent replicates were performed for each iron source and concentration.

Carbon source

The effects of three carbon sources on rooting and acclimatization were compared: sucrose, D-glucose, and D-fructose. The final concentration in the root expression medium was 83.2 mM, with 48 microshoots being scored per genotype and replica. The same variables as in rhizogenesis were measured. Survival was assessed after 6 weeks and expressed as percentage. Three independent replicates were performed for each experiment. In addition, seven individual vitroplants were randomly selected per genotype after 7 weeks of hardening, to determine the number of roots, length of the longest root, and the net stem elongation after potting. Stem length was measured from just above the basal callus to the apical shoot tip.

DNA extraction and microsatellite analysis

To evaluate clonal fidelity of rooted shoots, eight microsatellite primer pairs were selected among those developed by Victory et al. (2006). Selection was based on the polymorphism detected in our material. Leaf genomic DNA was extracted with the Qiagen DNeasy Plant Mini kit, according to the manufacturer's instructions (Qiagen, USA). Polymerase chain reaction (PCR) amplifications were performed in a final volume of 10 μl containing 1 μl 10 × reaction buffer (1× was 75 mM Tris–HCl, pH 9, 50 mM KCl, 2 mM $MgCl_2$ and 20 mM ammonium sulphate), 10 ng genomic DNA, 0.5 μM each primer, 200 μM each dNTP, and 0.4 units *Taq* DNA polymerase (Biotools B&M Labs, Spain). Reaction cycles consisted of an initial step of 5 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at the annealing temperature of each primer pair (based on Victory et al. 2006), and 30 s at 72 °C. After the 30th cycle, an additional extension step was performed for 10 min at 72 °C. Forward primers were labeled with fluorophores 6-FAM, PET, VIC, and NED (Applied Biosystems, USA) and PCR products were fractionated by capillary electrophoresis using an ABI 3730 Analyzer (Applied Biosystems). Fragment sizes were assessed with the Peak Scanner 1.0 software from Applied Biosystems. To ensure consistent results, three independent amplifications were performed per sample.

Histological analysis

Transversal sections of the basal part of rooted plants were obtained using a vibratome (Vibratome 1000 Plus, USA).

Selected fresh sections were directly stained in an aqueous solution of 0.02 % (w/v) toluidine blue for 2–3 min and rinsed briefly in water. Stomatal morphology was analyzed in the abaxial epidermis of acclimatized plants as well as microshoots kept on multiplication medium for 5 weeks. In both cases, leaf blade samples were taken between secondary veins, mounted with a droplet of 1:1 (v/v) glycerol-PBS (1.9 mM NaH_2PO_4, 8.4 mM Na_2HPO_4, 0.17 M NaCl), and observed under a fluorescent microscope (Zeiss Axiophot) coupled to a DFC 300FX Leica camera.

Statistical analysis

To minimize positional effects in growth chambers and the greenhouse, vessels and pots were distributed at random and moved weekly. Two subcultures were performed between treatments to avoid carryover effects. Three independent replicates were conducted per treatment for all experiments. A bifactorial design was used to detect hypothetical interactions between the genotype and the factor analyzed. Analysis of variance (ANOVA) was used to determine statistical significance, and the Fisher's least significant difference (LSD) was used as a post hoc test for pattern detection. Percentage data were earlier processed according to arcsin values.

Results and discussion

Effects of PG on in vitro growth, rooting, and survival

For all walnut genotypes analyzed, we found that microshoot performance (length, number of nodes, callus weight) was significantly enhanced by the addition of PG to the culture media (pre-rooting phases). Table 1 presents data for nine genotypes showing distinct responsiveness to PG compared with control treatments. Of all variables measured before rooting, the effects on callus size were particularly conspicuous. With respect to untreated explants, biomass gains ranged from approximately two-fold at 0.2 mM to five-fold at 0.8 mM. Statistically significant differences between treated and untreated explants were also found for internodal distances, nodes per microshoot, and microshoot length (Table 1). The observed responses were largely dose-dependent, with more pronounced effects being recorded at higher PG concentrations. The ultimate reasons for the in vitro effects of this compound remain little understood (Teixeira da Silva et al. 2013). PG and structurally-similar phenolic compounds have been reported to stimulate phenomena as diverse as bud induction in pepper (Kumar et al. 2005), shoot proliferation in potato (Sarkar and Naik 2000) or somatic embryogenesis in fir (Find et al. 2002).

Our results are indicative that PG has a beneficial effect on walnut micropropagation, at least for the hybrid under study. Compared with untreated material, the most striking observable difference was the massive swelling of stem portions in contact with the culture medium (Fig. 1). Basal calli were completely formed in about 2 weeks and then shoot growth started. Two findings suggest that the formation of a basal callus is critical at this stage: (1) the only explants that could be successfully established were those forming visible calli; and (2) during multiplication, there was a positive correlation between microshoot length and callus size ($r = 0.89$ for 64 measurements). Driver and Kuniyuki (1984) had reached the same conclusion during the in vitro establishment of Paradox walnut. Whereas the highest PG concentration tested (0.8 mM) was the most efficient in promoting growth, it also caused noticeable chlorosis and defoliation at the end of the subculture period. Likewise, PG treatments diminished in vitro rooting in the nine genotypes analyzed (Table 1). Although some genotypes were more sensitive than others, dose–response effects were generally observed, with higher PG concentrations resulting in lower rooting rates. Root formation was indeed abolished in most genotypes at 0.8 mM PG (Table 1). At this concentration, the mildest response was recorded for genotype J4, which showed a 50 % reduction in rooting ability compared to untreated samples. These results suggest that PG may have deleterious effects on rhizogenesis at sufficiently high concentrations. Leslie et al. (2005) found nonetheless a stimulating effect of supplementing their multiplication medium with 1 mM PG before root induction. Differences in genotype and/or culture conditions are likely causes of such variability. In line with this notion, Teixeira da Silva et al. (2013) describe several instances where apparently contradictory effects have been reported for PG and other phenolic compounds. Indeed, divergent cultivar-dependent responses to PG treatment have been reported in apple (Zimmerman 1984). Interestingly, De Klerk et al. (2011) observed different effects of PG on rooting depending on the specific auxin applied. Analogous results were reported for ferulic acid, another phenolic compound.

Our quantitative measurements suggest that vitroplant mortality during acclimatization depends largely on genotype and the concentration of PG used; both factors together explain 79.1 % of total variance. However, we did not find any clear correlation between mortality and the number of roots formed, except that vitroplants with a single root survived less than those with two or more roots for all nine genotypes. When microshoots were cultured without PG, survival rates were as high as 79 % (genotype J6). A drastic reduction of such numbers was nonetheless registered when PG was applied at concentrations above 0.2 mM. While further research is obviously needed, our

Table 1 Effects of different PG concentrations on walnut microshoot growth, rooting and survival

Genotype	PG (mM)	Callus weight (g)	Microshoot length (mm)	Nodes per microshoot	Rooting (%)	Roots per microshoot	Survival (%)
J1	0	0.37 ± 0.07^{a}	26.7 ± 7.6^{a}	9.1 ± 0.9^{a}	84.4 ± 9.2^{c}	3.5 ± 0.4^{b}	69.9 ± 6.5^{b}
	0.2	0.56 ± 0.06^{b}	30.5 ± 2.9^{b}	9.7 ± 2.2^{a}	81.1 ± 3.3^{c}	4.8 ± 0.3^{c}	64.2 ± 8.8^{b}
	0.4	0.89 ± 0.10^{c}	38.2 ± 6.2^{d}	11.1 ± 1.8^{b}	83.5 ± 4.0^{c}	2.9 ± 0.7^{ab}	29.3 ± 8.6^{a}
	0.6	0.99 ± 0.14^{d}	40.1 ± 4.4^{c}	11.0 ± 1.6^{b}	53.6 ± 5.6^{b}	2.2 ± 0.2^{a}	32.0 ± 7.4^{a}
	0.8	1.17 ± 0.17^{e}	41.2 ± 3.4^{c}	11.4 ± 0.6^{b}	33.3 ± 8.9^{a}	2.4 ± 0.6^{a}	28.8 ± 5.3^{a}
J2	0	0.19 ± 0.06^{a}	19.4 ± 5.0^{a}	1.9 ± 0.2^{a}	82.1 ± 6.6^{c}	2.1 ± 0.7^{a}	68.6 ± 5.7^{c}
	0.2	0.33 ± 0.08^{b}	22.6 ± 6.5^{a}	2.5 ± 0.4^{b}	71.5 ± 7.9^{b}	2.5 ± 0.4^{a}	54.1 ± 8.8^{b}
	0.4	0.53 ± 0.18^{c}	35.5 ± 3.3^{b}	2.3 ± 0.7^{ab}	68.0 ± 6.2^{b}	3.4 ± 0.6^{b}	44.5 ± 5.1^{b}
	0.6	0.78 ± 0.15^{d}	38.2 ± 7.6^{b}	2.6 ± 0.6^{b}	43.3 ± 6.5^{a}	2.6 ± 0.4^{a}	29.9 ± 6.4^{a}
	0.8	NE	NE	NE	NE	NE	NE
J3	0	NE	NE	NE	NE	NE	NE
	0.2	0.22 ± 0.03^{a}	17.6 ± 4.3^{a}	6.6 ± 0.7^{a}	NE	NE	NE
	0.4	0.29 ± 0.03^{b}	21.4 ± 1.3^{b}	7.2 ± 1.1^{b}	NE	NE	NE
	0.6	0.29 ± 0.04^{b}	20.9 ± 2.4^{b}	7.5 ± 0.6^{b}	NE	NE	NE
	0.8	0.38 ± 0.09^{c}	20.8 ± 1.6^{b}	7.5 ± 0.5^{b}	NE	NE	NE
J4	0	0.16 ± 0.05^{a}	19.6 ± 2.9^{a}	7.4 ± 0.3^{a}	81.1 ± 10.3^{d}	1.9 ± 0.4^{a}	79.0 ± 9.1^{c}
	0.2	0.29 ± 0.06^{b}	21.5 ± 0.9^{a}	8.1 ± 1.1^{b}	60.1 ± 7.0^{c}	2.1 ± 0.3^{a}	71.8 ± 5.2^{bc}
	0.4	0.46 ± 0.10^{c}	25.5 ± 1.2^{b}	9.4 ± 1.0^{c}	53.5 ± 4.9^{bc}	2.0 ± 0.2^{a}	60.3 ± 8.3^{b}
	0.6	0.55 ± 0.17^{d}	30.6 ± 3.8^{c}	9.7 ± 1.2^{c}	43.8 ± 9.9^{a}	2.9 ± 0.8^{b}	41.6 ± 7.3^{a}
	0.8	0.76 ± 0.16^{b}	36.7 ± 5.6^{d}	10.3 ± 1.3^{d}	43.1 ± 7.7^{a}	2.3 ± 0.6^{ab}	42.7 ± 4.4^{a}
J5	0	0.22 ± 0.01^{a}	21.1 ± 7.2^{a}	1.8 ± 0.4^{a}	92.1 ± 10.6^{c}	2.0 ± 0.5^{a}	78.8 ± 7.7^{c}
	0.2	0.43 ± 0.14^{b}	32.6 ± 4.5^{b}	3.6 ± 0.4^{b}	89.5 ± 7.1^{c}	2.9 ± 0.8^{bc}	84.3 ± 5.8^{c}
	0.4	0.63 ± 0.17^{c}	36.9 ± 9.3^{b}	3.3 ± 0.3^{b}	70.0 ± 8.1^{b}	3.2 ± 1.1^{c}	54.5 ± 9.7^{b}
	0.6	0.82 ± 0.20^{d}	42.1 ± 6.8^{c}	3.6 ± 0.9^{b}	58.7 ± 5.6^{a}	2.6 ± 0.8^{ab}	33.3 ± 4.4^{a}
	0.8	NE	NE	NE	NE	NE	NE
J6	0	0.14 ± 0.04^{a}	16.4 ± 2.2^{a}	5.9 ± 0.7^{a}	72.6 ± 5.2^{a}	1.9 ± 0.4^{a}	79.0 ± 6.8^{c}
	0.2	0.33 ± 0.16^{b}	21.4 ± 4.9^{b}	7.7 ± 1.3^{b}	61.4 ± 6.1^{a}	2.6 ± 0.9^{b}	61.2 ± 8.6^{b}
	0.4	0.55 ± 0.13^{c}	23.7 ± 2.7^{c}	8.5 ± 0.9^{c}	61.4 ± 8.2^{a}	2.7 ± 0.5^{b}	44.6 ± 8.1^{a}
	0.6	0.64 ± 0.12^{d}	22.5 ± 3.8^{bc}	8.5 ± 1.0^{c}	55.4 ± 7.3^{a}	2.3 ± 0.6^{ab}	NE
	0.8	0.65 ± 0.16^{d}	22.7 ± 4.5^{bc}	8.7 ± 0.9^{c}	NE	NE	NE
J7	0	0.25 ± 0.07^{a}	23.4 ± 5.9^{a}	8.8 ± 1.5^{a}	74.5 ± 6.8^{c}	1.1 ± 0.3^{a}	56.2 ± 9.5^{c}
	0.2	0.48 ± 0.13^{b}	29.0 ± 6.6^{b}	9.8 ± 0.5^{b}	59.9 ± 9.5^{b}	1.5 ± 0.9^{a}	44.4 ± 8.4^{b}
	0.4	0.80 ± 0.20^{c}	36.6 ± 3.3^{c}	10.6 ± 0.7^{c}	14.5 ± 3.1^{a}	1.7 ± 0.4^{a}	17.7 ± 5.6^{a}
	0.6	1.04 ± 0.16^{d}	41.9 ± 7.5^{d}	11.0 ± 1.2 ^{cd}	NE	NE	NE
	0.8	1.17 ± 0.24^{e}	41.8 ± 4.3^{d}	11.3 ± 1.6^{d}	NE	NE	NE
J8	0	0.14 ± 0.07^{a}	13.9 ± 4.1^{a}	6.3 ± 2.2^{a}	95.2 ± 9.3^{b}	3.1 ± 0.5^{a}	36.3 ± 6.9^{b}
	0.2	0.52 ± 0.15^{b}	29.2 ± 3.8^{b}	9.1 ± 0.9^{b}	84.1 ± 7.1^{ab}	3.7 ± 1.3^{a}	30.6 ± 10.1^{ab}
	0.4	0.72 ± 0.20^{c}	35.0 ± 7.1^{c}	9.9 ± 0.9^{c}	55.4 ± 6.1^{a}	2.7 ± 1.0^{a}	19.4 ± 7.4^{a}
	0.6	0.89 ± 0.20^{d}	36.9 ± 6.2^{c}	10.1 ± 0.6^{c}	66.2 ± 1.7^{a}	3.3 ± 1.8^{a}	NE
	0.8	1.11 ± 0.17^{e}	43.8 ± 4.2^{d}	10.2 ± 0.4^{c}	NE	NE	NE
J9	0	0.12 ± 0.05^{a}	14.1 ± 2.3^{a}	7.8 ± 0.4^{a}	75.9 ± 10.0^{c}	2.3 ± 0.2^{ab}	77.7 ± 7.3^{d}
	0.2	0.32 ± 0.10^{b}	23.8 ± 3.3^{b}	8.7 ± 0.7^{b}	66.7 ± 8.1^{c}	2.0 ± 0.8^{a}	61.6 ± 4.4^{c}
	0.4	0.53 ± 0.19^{c}	31.4 ± 3.8^{c}	10.4 ± 0.9^{c}	50.1 ± 2.8^{b}	2.8 ± 1.1^{b}	31.7 ± 6.7^{b}
	0.6	0.73 ± 0.13^{d}	33.6 ± 5.8 ^{cd}	10.5 ± 0.3^{c}	41.1 ± 7.4^{b}	2.7 ± 0.7^{b}	23.1 ± 9.7^{b}
	0.8	0.90 ± 0.23^{e}	37.7 ± 6.3^{d}	10.9 ± 0.5^{c}	11.1 ± 3.4^{a}	1.8 ± 0.5^{a}	NE

Values are means ± standard deviations. Superscript letters refer to responses that were significantly different ($p \leq 0.05$, LSD test). Microshoot growth was monitored during the sixth week of culture. Rooting was evaluated after 3 weeks and survival was assessed during the sixth week of acclimatization. Glucose was added during root formation. Non-evaluable treatments are indicated by NE

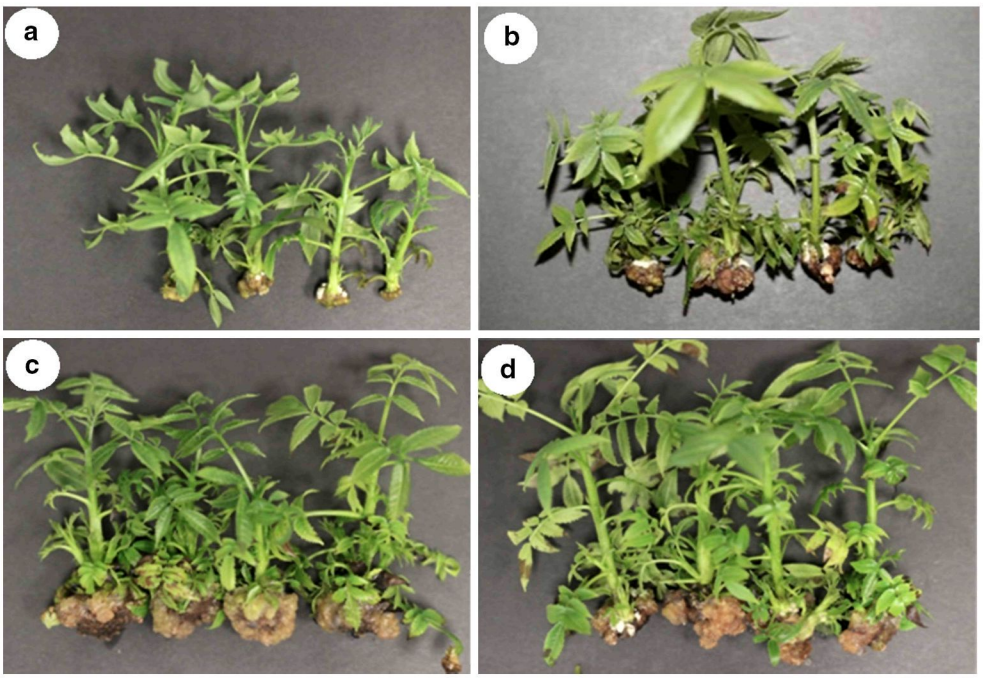

Fig. 1 Effects of PG on microshoot biomass and basal calli formation. Representative pictures are shown for four J6 microshoots (see Table 1). Analogous differences were observed for the remaining genotypes tested. Microshoots were either untreated (**a**) or treated with increasing amounts of PG: 0.2 mM (**b**), 0.4 mM (**c**) and 0.8 mM (**d**)

results demonstrate that moderate PG concentrations during shoot multiplication (0.4 mM), reduced at 0.2 mM 6 weeks before root induction, significantly promote walnut in vitro performance, without affecting rooting efficiency and plant survival in a substantial manner.

Effects of the iron chelate

The most common iron source for *Juglans* micropropagation is FeEDTA. At the concentration typically used in DKW medium (equivalent to 6.81 mg/L Fe^{2+}; McGranahan et al. 1987), this iron chelate did not prevent the appearance of chlorotic lesions under our experimental conditions, especially during the multiplication phase. Ashrafi et al. (2010) have reported similar findings for other walnut genotypes. Increasing FeEDTA levels up to 1.5 times (10.21 mg/L Fe^{2+}) did not improve microshoot performance. Rather, significant reductions were recorded for average shoot length and internodal distances (Table 2). The number of nodes per explant, however, was essentially unaffected, suggesting that chelate excess blocked internodal elongation. In line with this notion, higher levels of FeEDTA completely stopped growth and prevented formation of large basal calli. Rooting was also clearly affected by this chelate, as microshoots cultured with the highest concentration tested (10.21 mg/L Fe^{2+}) failed to form roots altogether. Even the lowest concentration caused a significant reduction in rooting percentage, down to as low as 1.1 % for J9. Compared with control, the number of roots per microshoot and the length of the longest root were reduced as well. Such adverse effects reverted when FeEDTA was replaced by FeEDDHA, which indeed improved microshoot quality at comparable iron concentrations (Table 2). Even at half the iron concentration provided with FeEDTA, the addition of FeEDDHA resulted in longer and healthier microshoots (Table 2), without any detectable deleterious effect. The most iron-sensitive genotype, J1, provided a good example: after 6 weeks of elongation, its average length in FeEDTA was less than half that in FeEDDHA (15.3 vs. 39.2 mm) and the same occurred for the average internodal length (1.42 vs. 3.46 mm). The beneficial effects of FeEDDHA also extended to rooting. As summarized in Table 2, significant improvements were recorded regarding the percentage of rooted microshoots, the number of roots per explant and also root length. Microshoot elongation was similarly boosted by this compound during root expression. Compared with FeEDTA, vitroplants obtained with FeEDDHA seemed healthier and much less affected by defoliation upon transplanting. While the reasons for the superiority of this iron chelate remain uncertain, our findings are indicative that the form in which iron is supplied is clearly important for walnut micropropagation. Similar conclusions have been reached for *Coryllus* (Nas and Read 2001), *Rosa* (van der Salm et al. 1994), and *Robus* (Zawadzka and Orlikowska 2009).

Table 2 Effects of FeEDTA and FeEDDHA on microshoot growth and rooting

Genotype	Iron source	Microshoot length (mm)	Nodes per microshoot	Rooting (%)	Roots per microshoot	Root length (mm)
J1	FsI	24.0 ± 4.5^{b}	10.5 ± 0.5^{a}	8.2 ± 2.3^{a}	1.1 ± 0.3^{b}	18.9 ± 6.3^{b}
	FsII	15.3 ± 2.1^{a}	10.9 ± 0.9^{ab}	NE	NE	NE
	FcI	29.8 ± 3.4^{c}	10.6 ± 0.7^{a}	77.0 ± 11.1^{b}	2.3 ± 0.5^{c}	31.9 ± 4.8^{c}
	FcII	39.2 ± 5.6^{d}	11.2 ± 1.1^{b}	88.8 ± 4.5^{b}	2.9 ± 0.4^{c}	34.3 ± 5.6^{c}
J2	FsI	21.8 ± 3.2^{a}	8.3 ± 0.9^{a}	21.3 ± 4.1^{b}	1.6 ± 0.5^{a}	13.6 ± 3.3^{b}
	FsII	12.7 ± 2.2^{a}	9.9 ± 0.7^{b}	8.8 ± 1.9^{a}	1.1 ± 0.2^{a}	8.5 ± 1.2^{a}
	FcI	33.3 ± 1.2^{c}	10.0 ± 0.8^{b}	53.3 ± 3.4^{c}	2.5 ± 0.6^{b}	16.6 ± 4.1^{b}
	FcII	36.1 ± 6.4^{c}	9.4 ± 0.5^{b}	59.8 ± 5.4^{c}	3.1 ± 0.9^{b}	25.5 ± 3.6^{c}
J3	FsI	15.7 ± 2.2^{a}	9.4 ± 0.8^{a}	NE	NE	NE
	FsII	15.6 ± 1.5^{a}	10.3 ± 0.6^{b}	NE	NE	NE
	FcI	20.0 ± 2.9^{b}	10.1 ± 0.8^{b}	23.3 ± 4.7^{a}	2.2 ± 0.8^{a}	23.5 ± 3.2^{a}
	FcII	21.9 ± 4.2^{c}	9.6 ± 0.4^{a}	45.9 ± 4.4^{b}	2.9 ± 1.1^{a}	29.0 ± 2.5^{b}
J4	FsI	23.3 ± 1.6^{a}	NE	8.7 ± 4.3^{a}	1.1 ± 0.9^{a}	6.5 ± 6.0^{a}
	FsII	NE	NE	NE	NE	NE
	FcI	NE	NE	NE	NE	NE
	FcII	24.1 ± 2.1^{a}	NE	31.9 ± 4.8^{b}	1.5 ± 0.6^{a}	14.1 ± 6.1^{b}
J5	FsI	26.6 ± 3.1^{b}	7.2 ± 1.1^{b}	43.2 ± 7.6^{a}	1.3 ± 0.7^{a}	12.1 ± 7.0^{a}
	FsII	12.8 ± 5.8^{a}	4.3 ± 0.3^{a}	NE	NE	NE
	FcI	30.1 ± 2.9^{c}	10.3 ± 0.9^{c}	74.4 ± 3.4^{b}	2.7 ± 0.4^{b}	19.5 ± 3.5^{b}
	FcII	37.9 ± 5.3^{d}	11.6 ± 0.5^{d}	91.5 ± 5.1^{c}	3.3 ± 0.4^{b}	27.3 ± 4.9^{c}
J6	FsI	16.5 ± 3.6^{a}	6.9 ± 0.8^{a}	19.3 ± 8.6^{a}	1.4 ± 0.7^{a}	11.4 ± 3.4^{a}
	FsII	NE	NE	NE	NE	NE
	FcI	NE	NE	NE	NE	NE
	FcII	22.3 ± 4.1^{b}	8.5 ± 0.8^{b}	62.2 ± 6.7^{c}	2.7 ± 0.9^{b}	21.8 ± 5.1^{b}
J7	FsI	24.6 ± 4.4^{b}	9.7 ± 0.5^{a}	18.3 ± 8.4^{b}	1.9 ± 0.7^{b}	14.3 ± 2.4^{b}
	FsII	18.4 ± 5.1^{a}	11.1 ± 0.7^{c}	NE	NE	NE
	FcI	33.1 ± 2.9^{c}	10.8 ± 0.4^{bc}	66.6 ± 6.1^{c}	2.1 ± 2.2^{b}	21.8 ± 6.7^{c}
	FcII	37.7 ± 1.8^{d}	10.2 ± 0.5^{ab}	70.1 ± 5.8^{c}	1.9 ± 1.1^{b}	28.8 ± 8.3^{d}
J8	FsI	31.2 ± 2.5^{a}	NE	54.5 ± 1.9^{a}	2.2 ± 0.9^{a}	20.5 ± 4.9^{a}
	FsII	NE	NE	NE	NE	NE
	FcI	NE	NE	NE	NE	NE
	FcII	40.8 ± 1.9^{b}	NE	74.4 ± 4.3^{b}	3.1 ± 0.6^{b}	25.7 ± 6.1^{b}
J9	FsI	17.8 ± 3.0^{b}	10.0 ± 0.7^{a}	11.5 ± 2.3^{a}	1.5 ± 0.7^{a}	14.3 ± 2.4^{b}
	FsII	14.8 ± 1.1^{a}	10.6 ± 0.7^{b}	NE	NE	NE
	FcI	26.1 ± 2.3^{c}	10.3 ± 0.6^{b}	32.6 ± 1.9^{b}	3.0 ± 1.3^{b}	24.5 ± 4.7^{c}
	FcII	31.4 ± 3.8^{d}	10.4 ± 0.9^{b}	50.1 ± 2.8^{c}	3.2 ± 1.1^{b}	31.7 ± 6.7^{d}

Values are means ± standard deviations. Superscript letters refer to responses that were significantly different ($p \leq 0.05$, LSD test). Microshoot growth was monitored during the sixth week of culture. Rooting was evaluated after 3 weeks (2 weeks for genotype J1). Sucrose was used as carbon source during root formation. Non-evaluable treatments are indicated by NE. FsI FeEDTA at 6.81 mg/L Fe^{2+}, FsII FeEDTA at 10.21 mg/L Fe^{2+}, FcI FeEDDHA at 3.40 mg/L Fe^{3+}, FcII FeEDDHA at 6.81 mg/L Fe^{3+}

Effects of the carbon source

We tested the effects of supplementing the root expression culture medium with three alternative carbon sources: glucose, fructose or sucrose. The latter is used in virtually all in vitro walnut protocols (e.g., McGranahan and Leslie 1988; Jay-Allemand et al. 1992; Dolcet-Sanjuan et al. 2004; Vahdati et al. 2004; Leslie et al. 2009). No clear pattern of influence on rhizogenesis emerged from these experiments, where genotype was identified as the major driver of in vitro behavior (it explains 76.5 % of the total variance). At early culture stages fructose typically

Table 3 Effects of alternative carbon sources on microshoot rooting and survival of vitroplants

Genotype	Carbon source	Rooting (%)	Roots per microshoot	Root length (mm)	Microshoot length (mm)	Survival (%)
J1	Fru	89.5 ± 7.8^{a}	4.5 ± 0.3^{a}	25.9 ± 12.2^{a}	43.1 ± 8.9^{a}	33.7 ± 8.9^{a}
	Glu	80.4 ± 10.0^{a}	4.8 ± 1.1^{a}	35.6 ± 18.5^{b}	45.3 ± 10.0^{a}	66.7 ± 10.2^{c}
	Suc	76.7 ± 20.8^{a}	4.4 ± 0.4^{a}	33.7 ± 18.7^{b}	42.7 ± 9.5^{a}	47.1 ± 10.4^{b}
J2	Fru	78.7 ± 5.2^{a}	3.0 ± 1.5^{a}	17.8 ± 7.6^{a}	25.4 ± 3.9^{a}	32.1 ± 8.5^{a}
	Glu	68.2 ± 4.9^{a}	2.1 ± 0.6^{a}	26.4 ± 5.1^{b}	29.1 ± 4.5^{a}	57.0 ± 7.4^{b}
	Suc	67.3 ± 5.1^{a}	2.2 ± 1.0^{a}	27.9 ± 6.5^{b}	28.7 ± 6.8^{a}	56.8 ± 9.1^{b}
J3	Fru	95.0 ± 7.1^{c}	6.1 ± 1.3^{b}	27.0 ± 7.4^{a}	21.1 ± 4.3^{a}	49.6 ± 8.1^{a}
	Glu	68.9 ± 8.9^{a}	3.1 ± 1.0^{a}	28.2 ± 8.8^{a}	28.6 ± 4.9^{b}	81.0 ± 7.4^{b}
	Suc	78.1 ± 7.1^{b}	3.5 ± 2.0^{a}	29.9 ± 12.7^{a}	29.4 ± 6.4^{b}	74.6 ± 6.2^{b}
J4	Fru	63.3 ± 3.2^{a}	5.0 ± 1.4^{b}	30.9 ± 4.6^{a}	24.2 ± 3.3^{a}	65.4 ± 5.5^{b}
	Glu	61.4 ± 3.9^{a}	3.1 ± 1.0^{a}	29.9 ± 4.1^{a}	26.5 ± 1.9^{b}	51.0 ± 4.3^{ab}
	Suc	48.5 ± 4.3^{a}	2.6 ± 1.0^{a}	29.2 ± 6.2^{a}	26.4 ± 2.4^{b}	46.2 ± 5.1^{a}
J5	Fru	91.8 ± 5.2^{a}	4.2 ± 0.9^{a}	20.5 ± 4.5^{a}	32.6 ± 3.9^{a}	75.7 ± 3.3^{a}
	Glu	90.1 ± 6.1^{a}	3.6 ± 0.5^{a}	24.4 ± 2.8^{a}	32.6 ± 5.5^{a}	83.3 ± 2.7^{a}
	Suc	95.2 ± 6.1^{a}	3.3 ± 0.8^{a}	24.7 ± 5.1^{a}	35.9 ± 4.0^{a}	76.3 ± 2.2^{a}
J6	Fru	89.5 ± 9.3^{b}	3.6 ± 1.3^{b}	33.3 ± 4.6^{b}	24.0 ± 3.4^{a}	62.1 ± 6.9^{a}
	Glu	67.1 ± 8.2^{a}	2.5 ± 0.8^{a}	27.8 ± 6.0^{a}	25.4 ± 2.8^{a}	65.3 ± 4.4^{a}
	Suc	65.2 ± 6.6^{a}	2.2 ± 1.2^{a}	25.8 ± 2.7^{a}	24.8 ± 2.1^{a}	55.6 ± 5.5^{a}
J7	Fru	79.3 ± 9.3^{b}	3.7 ± 1.6^{b}	28.1 ± 5.4^{a}	24.0 ± 3.4^{a}	43.6 ± 4.3^{a}
	Glu	64.6 ± 7.2^{ab}	2.4 ± 0.4^{a}	34.9 ± 6.7^{b}	25.4 ± 2.8^{a}	49.2 ± 7.4^{a}
	Suc	55.6 ± 6.6^{a}	2.5 ± 0.9^{a}	29.2 ± 8.1^{a}	24.8 ± 2.1^{a}	51.5 ± 6.5^{a}
J8	Fru	78.6 ± 8.7^{a}	4.0 ± 0.8^{b}	20.7 ± 4.2^{a}	28.9 ± 3.0^{a}	28.3 ± 1.5^{a}
	Glu	77.8 ± 4.8^{a}	3.0 ± 0.6^{a}	21.5 ± 4.2^{a}	30.0 ± 4.3^{ab}	32.7 ± 1.1^{a}
	Suc	82.6 ± 7.6^{a}	3.4 ± 0.7^{ab}	19.6 ± 3.7^{a}	32.3 ± 1.7^{b}	26.5 ± 2.7^{a}
J9	Fru	73.5 ± 6.6^{b}	3.4 ± 1.4^{a}	25.5 ± 5.7^{a}	28.9 ± 3.9^{a}	56.8 ± 5.6^{a}
	Glu	64.3 ± 10.5^{ab}	3.1 ± 1.4^{a}	28.5 ± 8.3^{a}	32.6 ± 5.5^{b}	74.1 ± 5.9^{b}
	Suc	49.5 ± 7.6^{a}	2.7 ± 1.5^{a}	28.5 ± 10.2^{a}	35.9 ± 4.0^{b}	68.8 ± 4.3^{ab}

Values are means ± standard deviations. Data for five genotypes are presented. Superscript letters refer to responses that were significantly different ($p \leq 0.05$, LSD test). Rooting was evaluated after 3 weeks and survival during the sixth week of acclimatization. FeEDDHA (6.81 mg/L of Fe^{3+}) was used as iron source and rooting medium was supplemented with PG (0.2 mM). All sugars were added to the rooting medium to a final concentration of 83.2 mM

Fru fructose, *Glu* glucose, *Suc* sucrose

produced the highest number of roots, especially for genotypes J6 and J9, but also the shorthest microshoots (Table 3). The opposite was observed for sucrose. Such genotype-specific variability continued at later stages, with higher survival rates being most often associated with the presence of glucose in the culture medium (except for genotype J4). Different sugar-dependent effects have been reported for micropropagated long-lived woody species (Custodio et al. 2004; da Rocha Corrêa et al. 2005; Al-Khateeb 2008; and others). Genotypic differences aside, we found that glucose is the most convenient sugar for walnut micropropagation under our experimental conditions. The variable effects reported here and in the aforementioned studies probably reflect the interplay between genotype-specific factors and the complex regulatory roles played by carbohydrates (reviewed by Rolland et al. 2006).

Improved rooting and acclimatization

Regarding microshoot rooting, we found statistically significant genotypic effects on all quantitative variables analyzed, namely, rooting percentage, root number, and length of the longest root. These variables were not clearly linked, as exemplified by genotype J4: despite forming rather long roots, it showed lower rooting ability than, for example, the J5 genotype (Table 3). Some measurements summarized in this table hinted at a possible link between microshoot length and in vitro rooting. The best example is provided by genotypes J1 and J5, which exhibited both the highest rooting percentages on glucose (80 and 90 %, respectively) and the longest microshoots (>32 mm after 3 weeks on rooting medium). However, this trend was not observed in the presence of fructose, as exemplified by genotype J3.

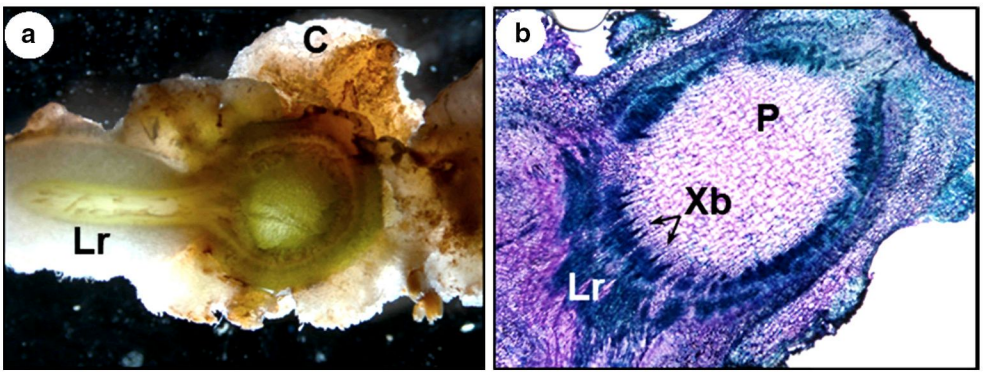

Fig. 2 Root morphology at the beginning of the acclimatization phase. **a** Microshoot basal view, showing a newly formed lateral root. **b** Toluidine blue-staining of basal stem sections evidenced the vascular connection between newly formed roots and the stem cambial tissue (×4 magnification with respect to **a**). *C* callus tissue, *Lr* lateral root, *P* pith, *Xb* xylem bundles

Table 4 Genetic stability of the MJ209xRA micropropagated material

Locus	Motif	Label	Allelic sizes
WGA004	$(GT)_5(GA)_{15}$	NED	231, 235, 261
WGA082	$(CT)_{20}$	6-FAM	171,177, 185
WGA090	$(CT)_4T(TC)_{14}$	6-FAM	134, 180
WGA147	$(GA)_{14}(GT)_8$	VIC	183,191
WGA148	$(AG)_{15}$	NED	247 261
WGA204	$(AG)_{15}$	VIC	168, 176, 187
WGA221	$(CT)_8$	PET	227, 232
WGA256	$(CT)_{19}$	PET	224, 240, 275
Number of primers	16		
Total alleles scored	224		
Intra-genotype polymorphisms scored	0		
Minimum number of alleles per genotype	8		
Maximum number of alleles per genotype	10		
Alleles detected per genotype (average)	8.7		

Eight microsatellite markers were used to assess the clonal fidelity of genotypes J1 to J9 after micropropagation. For each genotype, leaf genomic DNA was extracted and amplified as described in Materials and Methods, from both the original mother tree and four randomly selected acclimatized plantlets. We used the primers described by Victory et al. (2006), labeled as indicated (forward primer). Products from multiplex amplifications were identified with an ABI 3730 Analyzer and Peak Scanner 1.0 software (Applied Biosystems). Three independent amplifications were performed per sample for consistency

Another interesting finding was that in vitro rooting did not clearly correlate with plant survival. Thus, J4 and J9 vitroplants survived better than J8 ones in the presence of glucose, despite that J8 rooted better (Table 3). Likewise, the highest survival rate on glucose was observed for J5 vitroplants, which did not outperform other genotypes in rooting. Besides genotypic effects, these observations suggest that plant survival is probably less correlated with rooting efficiency than with the physiological state reached during in vitro culture. In agreement with this hypothesis, differences in rooting attributable to the carbon source did not translate into survival rates: despite producing more roots on fructose than on glucose or sucrose, microshoots survived less on the former sugar (Table 3). In any case, poorly rooted microshoots were prone to die during acclimatization.

On average, microshoots exposed to sucrose or glucose were significantly taller than those exposed to fructose. They also showed more newly-formed roots, which tended to replace those formed in vitro (data not shown). Genotypic effects aside, sugar choice influenced plant performance even after 7 weeks of acclimatization: the net length increase of J9 vitroplants, for example, was 28.1 ± 2.4 mm for glucose and 18.4 ± 1.9 mm for sucrose, while it was only 12.4 ± 1.5 mm for fructose. Analogous differences, in percentage, were recorded for other genotypes. Our findings are in agreement with previous results indicating that soluble sugars can substantially influence cell division and morphogenesis (Gibson 2005; Rolland et al. 2006). Not only roots were weaker upon fructose exposure, but the root system was more compact altogether. Glucose or sucrose

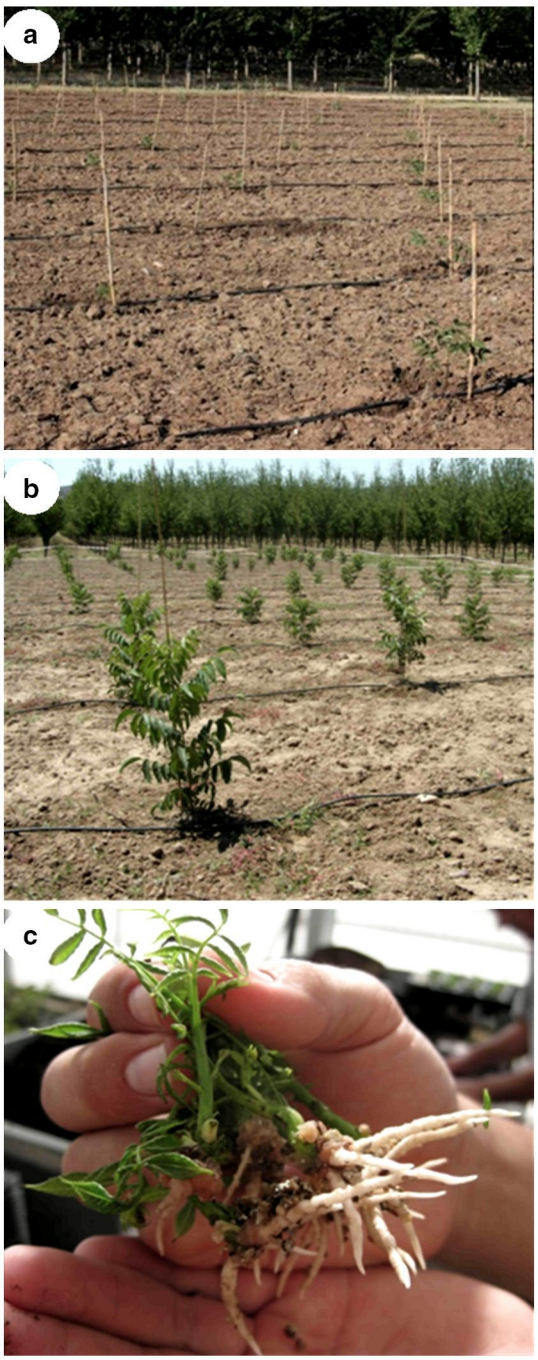

Fig. 3 Clonal plantation in El Soto (Toledo, Spain) of selected MJ209 × RA genotypes micropropagated as described in this paper. Pictures were taken in May 2014 (**a**) and July 2014 (**b**). **c** Close-up of the root system. To date, over 5800 clonal walnuts have been successfully planted

favored lateral growth along horizontal planes, giving rise to wider and thicker radical systems. These observations may help explain why explants exposed to fructose were less apt to withstand acclimatization (Table 3).

Since incomplete vascular connection of newly formed roots can be a major cause of walnut microshoot mortality (Driver and Kuniyuki 1984), we performed root histological analyses during the acclimatization phase. These confirmed the physical continuity between emerging roots and stem cambial tissue for all genotypes tested (Fig. 2). Newly formed roots were thus independent of basal calli, a prerequisite for vitroplants to be self-sufficient. Moreover, the normal organization of root xylem tissue (Fig. 2b) was indicative of correct root functionality. The same is true for in vitro formed leaves, which harbored normal stomata and were not spontaneously shed upon acclimatization (not shown). In summary, walnut in vitro rooted shoots did not present any morphological anomalies that might promote mortality during acclimatization.

Genetic profiling and stability

Since plants regenerated in vitro may suffer culture-induced genetic changes (Philips et al. 1994), we selected eight microsatellite loci to assess the genetic stability of the micropropagated material (Table 4). These loci were specifically selected on the basis of their discriminant power towards a large sample of the original MJ209xRA seedling plantation (50 individual trees), including all genotypes under study. For each clonal line, we compared the microsatellite profile of four randomly chosen acclimatized plantlets with that of the *mother* tree (i.e., genotype). Interestingly, not a single case of polymorphism was recorded in these experiments, supporting (1) the clonal nature of the propagated material and (2) that the experimental conditions described here do not cause major genetic instability. Somaclonal variation cannot be excluded, however, unless whole genomes are analyzed. In any case, the fact that our protocol does not involve a callus phase should significantly reduce this possibility (Carra et al. 2012).

Conclusions

The goal of this study was to improve current protocols for walnut micropropagation. An optimal combination of FeEDDHA and PG is described that results in significant enhancement of microshoot rooting and plant survival during acclimatization, compared to previous reports. Our data also indicate that genotype is a major factor influencing the overall success of walnut micropropagation. Selection programs aimed at mass production of elite genotypes must pay attention to these aspects, making fine adjustments when necessary. By following this strategy, we have successfully planted over 5800 hybrid walnut trees belonging to the genotypes studied here in different locations of Spain (Fig. 3 shows a representative example). Four additional genotypes are currently being planted.

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Authors contribution statement R.L., A.C. and A.V.M. conducted all in vitro and acclimatization experiments. I.U. contributed to genotype selection and data collection. M.D. participated in data analysis. R.L. and L.G. designed the study and wrote the paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Aletá N, Ninot A, Voltas J (2004) Retrospective evaluation of parental selection in nursery tests of *Juglans regia* L. using a mixed model analysis. Silvae Genet 53:26–32

Al-Khateeb AA (2008) Regulation of in vitro bud formation of date palm (*Phoenix dactylifera* L.) cv. Khanezi by different carbon sources. Bioresour Technol 99:6550–6555

Ashrafi EN, Vahdati K, Ebrahimzadeh H, Mirmasoumi M (2010) Analysis of in vitro explants mineral contents to modify medium mineral composition for enhancing growth of Persian walnut (*Juglans regia* L.). J Food Agric Environ 8:325–329

Becquey J (1997) Les noyers à bois. Institut pour le Développement Forestier, Paris

Bosela MJ, Michler CH (2008) Media effects on black walnut (*Juglans nigra*) shoot growth in vitro: evaluation of multiple nutrient formulations and cytokinin types. In vitro Cell Dev Biol Plant 44:316–329

Carra A, Sajeva M, Abbate L, Siragusa M, Sottile F, Carimi F (2012) In vitro plant regeneration of caper (*Capparis spinosa* L.) from floral explants and genetic stability of regenerants. Plant Cell Tissue Organ Cult 109:373–381

Clark J, Hemery G (2010) Walnut hybrids in the UK: fast growing quality hardwoods. Q J For 104:43–46

Cornu D, Jay-Allemand C (1989) Micropropagation of hybrid walnut trees (*Juglans nigra* × *Juglans regia*) through culture and multiplication of embryos. Ann Sci For 46S:113–116

Custodio L, Martins-Loução MA, Romano A (2004) Influence of sugars on in vitro rooting and acclimatization of carob tree. Biol Plant 48:469–472

da Rocha Corrêa L, da Rocha Corrêa L, Paim DC, Schwambach J, Fett-Neto AG (2005) Carbohydrates as regulatory factors on the rooting of *Eucalyptus saligna* Smith and *Eucalyptus globulus* Labill. Plant Growth Regul 45:63–73

De Klerk GJ, Guan H, Huisman P, Marinova S (2011) Effects of phenolic compounds on adventitious root formation and oxidative decarboxilation of applied indolacetic acid in *Malus* Jork 9. Plant Growth Regul 63:175–185

Dolcet-Sanjuan R, Claveria E, Gruselle R, Meier-Dinkel A, Jay-Allemand C, Gaspar T (2004) Practical factors controlling in vitro adventitious root formation from walnut shoots micro-cuttings. J Am Soc Hortic Sci 129:198–203

Driver JA, Kuniyuki AH (1984) In vitro propagation of Paradox walnut rootstock. Hortic Sci 19:507–509

Find J, Grace L, Krogstrup P (2002) Effect of anti-auxins on maturation of embryogenic tissue cultures of Nordmanns fir (*Abies nordmanniana*). Physiol Plant 116:231–237. doi:10.1034/j.1399-3054.2002.1160213.x

Gibson SI (2005) Control of plant development and gene expression by sugar signaling. Curr Opin Plant Biol 8:93–102

Jay-Allemand C, Capelli P, Cornu D (1992) Root development of in vitro hybrid walnut microcuttings in a vermiculite-containing gelrite medium. Sci Hortic 51:335–342

Kumar V, Gururaj HB, Narasimha Prasad BC, Giridhar P, Ravishankar GA (2005) Direct shoot organogenesis on shoot apex from seedling explants of *Capsicum annuum* L. Sci Hortic 106:237–246

Leal DR, Sánchez-Olate M, Avilés F, Materan ME, Uribe M, Hasbún R, Rodríguez R (2007) Micropropagation of *Juglans regia* L. In: Jain SM, Häggman H (eds) Protocols for micropropagation of woody trees and fruits. Springer, Heidelberg, pp 381–390

Leslie CA, Hackett WP, Bujazha D, Hirbod S, McGranahan GH (2005) Adventitious rooting and clonal plant production of hybrid walnut (*Juglans*) rootstock selections. Acta Hortic 705:325–328

Leslie CA, Hackett WP, McGranahan GH (2009) Improved rooting methods for walnut (*Juglans*) microshoots. Acta Hortic 861:365–372

McGranahan G, Leslie CA (1988) In vitro propagation of mature Persian walnut cultivars. Hortic Sci 23:220

McGranahan GH, Driver JA, Tulecke W (1987) Tissue culture of *Juglans*. In: Bonga JM, Durzan DJ (eds) Cell and tissue culture in forestry, vol 3. Martinus Nijhoff, Boston, pp 261–271

Moreno RJL, Morales AV, Daquinta M, Gomez L (2012) Towards scaling-up the micropropagation of *Juglans major* (Torrey) Heller var. 209 × *J. regia* L., a hybrid walnut of commercial interest. In: Park YS, Bonga JM (eds) Integrating vegetative propagation, biotechnologies and genetic improvement for tree production and sustainable forest management. IUFRO, Czech Republic, pp 80–91

Nas MN, Read PE (2001) Micropropagation of hybrid hazelnut: medium composition, physical state and iron source affect shoot morphogenesis, multiplication and explant vitality. Acta Hortic 556:251–258

Phan CT, Hegedus P (1985) Possible metabolic basis for the developmental anomaly observed in in vitro culture, called 'vitreous plants'. Plant Cell Tissue Org Cult 6:83–94

Philips RL, Kaeppler SM, Olhoft P (1994) Genetic instability of plant tissue cultures: breakdown of normal control. Proc Natl Sci USA 91:5222–5226

Rolland F, Baena-Gonzalez E, Sheen J (2006) Sugar sensing and signaling in plants: conserved and novel mechanisms. Ann Rev Plant Biol 57:675–709

Sarkar D, Naik PS (2000) Phloroglucinol enhances growth and rate of axillary shoot proliferation in potato shoot tip cultures in vitro. Plant Cell Tissue Organ Cult 60:139–149

Tallón CI, Porras I, Pérez-Tornero O (2012) Efficient propagation and rooting of three citrus rootstocks using different plant growth regulators. In vitro Cell Dev Biol Plant 48:488–499

Teixeira da Silva JA, Dobránzki J, Ross S (2013) Phloroglucinol in plant tissue culture. In vitro Cell Dev Biol Plant 49:1–16

Toosi S, Dilmagani K (2010) Proliferation of *Juglans regia* L. by in vitro embryo culture. J Cell Biol Genet 1:12–19

Vahdati K, Leslie C, Zamani Z, McGranahan G (2004) Rooting and acclimatization of in vitro-grown shoots from mature trees of three persian walnut cultivars. Hortic Sci 39:324–327

Vahdati K, Razaee R, Mirmasoomi M (2009) Micropropagation of some dwarf and early mature walnut genotypes. Biotechnology 8:171–175

Van der Salm TPM, Van der Toorn CJG, Hänish Ten Cate CH, Dubois LAM, De Vries DP, Dons HJM (1994) Importance of the iron chelate formula for micropropagation of *Rosa hybrida* L. "Moneyway". Plant Cell Tissue Organ Cult 37:73–77

Victory ER, Glaubitz JC, Rhodes OE, Woeste KE (2006) Genetic homogeneity in *Juglans nigra* (Juglandaceae) at nuclear microsatellites. Am J Bot 93:118–126

Woeste K, Michler C (2011) *Juglans*. In: Chittaranjan K (ed) Forest trees Wild crop relatives: genomic and breeding resources. Springer, Berlin, pp 77–88

Zawadzka M, Orlikowska T (2009) Influence of FeEDDHA on in vitro rooting and acclimatization of red raspberry (*Rubus idaeus* L.) in peat and vermiculite. J Hortic Sci Biotechnol 84:599–603

Zimmerman RH (1984) Rooting apple cultivars in vitro: interactions among light, temperature, phloroglucinol and auxin. Plant Cell Tissue Organ Cult 3:301–311